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Genotoxic Effects of Erioflorin Acetate and Erioflorin Methacrylate: Sesquiterpene Lactones Isolated from *Podanthus ovatifolius* Lag. (Compositae)

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Chemotherapy of human malignancies has increased in efficiency during last decades, in spite of the fact that secondary undesirable effects on normal cells have not been left out. These effects frequently include genetic damage because DNA organization or chromosome distribution are modified. Thus, paradoxically, mutagenic, carcinogenic, and teratogenic risks are increased in patients treated with anticancer drugs. Improved drug therapy would only be recommended when the risk-benefit evaluation makes it appropriated.

Three major sesquiterpene lactones: erioflorin methacrylate (EM), erioflorin acetate (EA), and ovatifolin acetate (OA) have been isolated from neutral extracts of Podanthus ovatifolius, and all of them have been shown to exhibit antineoplastic properties as tested in standard KB human epidermoid carcinoma of the nasopharynx assay procedures carried out in National Cancer Institute (N.I.H.) U.S.A. These experiments showed the following LD50: EM = 0.69 g/ml, EA = 1.80 g/ml and OA = 250 µg/ml. These chemicals were isolated as part of a screening program of Chilean plants for anti-tumour activity. (Bhakuni et al. 1976; Gnecco et al. 1973).

In the present study we report in vivo induction of micronuclei in bone-marrow mice polychromatic erythrocytes (MPCE) by EM, and EA (Fig. 1). The micronucleus test developed by Schmid and coworkers, (1975), is an alternative to cytogenetic studies on bone-marrow in vivo. Chemicals can be screened for chromosome breaking ability by measuring the frequency of erythrocytes with micronuclei derived from acentric chromosomal fragments or lagging chromosomes (Heddle and Carrano, 1977). Due to its simplicity and wide range of applicability, the test has been used extensively as one of the major short-term tests for evaluating mutagenicity and carcinogenicity (Wild, 1978; Bruce and Heddle, 1979; Tsuchimoto and Matter, 1979; Jensen and Ramel, 1980; Cea et al. 1983; Heddle, 1983; Cea et al. 1986; Alarcón et al. 1986). This assay has also been described for genotoxicity studies in several mammalian tissues other than polychromatic erythrocytes, such as peripheral lymphocytes (Countryman and Heddle, 1976), liver (Tates et al. 1980), colon epithelium

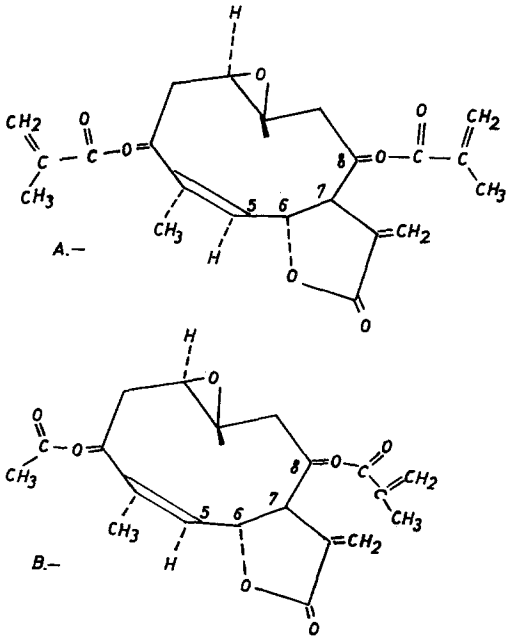

Figure 1. A.- Erioflorin Methacrylate B.- Erioflorin acetate

(Goldberg et al. 1983; Duncan et al. 1983), and spermatids (Lahdetie and Parvinen, 1981; Parvinen et al. 1984; Toppari et al. 1986) and it correlated well with the incidence of chromosome aberrations in bone-marrow cell (Goetz et al. (1975).

MATERIALS AND METHODS

EM ($C_{23}H_{28}O_7$) and EA ($C_{21}H_{22}O_7$) were kindly supplied by Dr. Mario Silva, Phytochemistry Laboratory, University of Concepción. Cytotoxicity tests for KB cells were carried out at the National Cancer Institute (N.I.H.), U.S.A.

The micronucleus test was carried out as proposed by Schmid (1975) but modified according to Das and Kar (1980). Two-month old male Balb/c mice (Molecular Biology Department Biotherium, University of Concepción) weighing ca. 20 g were given a single drug dose (0.2 ml) diluted in dioxan-distilled water (1:10) via i.p. injection (1,4-Dioxan p.a. Merck). Four doses for each chemical were selected on the basis of the KB cells LD50. Doxorubicine (Adriamycine, Farmitalia) at 10.0 µg/g b.wt. was used as positive control and a mixture of dioxan-distilled water as negative control.

Four mice per group in each experiment were sacrified 30 h. after injection. Femurs were prepared for bone-marrow micronucleus test by the method of Oliver and Goldstein (1978). Slides were stained with May-Grünwald and Giemsa according to the schedule outlined by Cole et al. (1979), which maximizes the

staining differences between polychromatic (PCE) and normochromatic (NCE) erythrocytes. About 5.000-8.000 PCE and NCE per animal per dose, were blind scored and those with micronuclei were recorded. The Mann-Whitney U test was employed for statistical analysis. The significance was tested at $p < 0.05$ level.

RESULTS AND DISCUSSION

The average quantitative data of the mice bone-marrow cells study are presented in Table 1. EM does not significantly increase MPCE incidence above that induced by the negative control (dioxan-water mixture) (Fig. 2 and Table 2).

At the two lower doses used, EA shows significantly lower MPCE incidences when compared with the incidences exhibited by the negative control and those exhibited by the two higher doses used. The frequencies of micronuclei observed in animals treated with the higher doses of EA are not different from those observed in the negative control (Fig. 3 and Table 3).

EA exhibits an *in vitro* KB cell LD50 which is threefold the EM LD50 for the same transformed nasopharingeal cells, showing that EM cells toxicity is higher. This level of toxicity agrees with PCE/NCE rate lower than those found in animals treated with higher doses of EM, which are significantly different from those found in negative controls (Fig. 2 and Table 2). No significant differences were observed between EA PCE/NCE rates and those of the negative control (Fig. 3 and Table 3).

On the other hand, both EM and EA at the two lower doses used show minor MNCE incidences when compared with the incidences of the negative controls (Figs. 2 and 3 and Tables 2 and 3). The other doses do not show higher MNCE incidence values than those of the negative controls. Also, at all doses, MNCE incidences are lower than the incidences of MPCE.

The micronuclei observed in all EM treatments were only single round-shaped with size of 1/5-1/6 of the cell diameter. EA treatments also showed single round-shaped micronuclei with size of 1/8 of the cell diameter (89.8%) but they also showed single oval-shaped micronuclei with 1/4 of the cell diameter. Multi-micronucleated erythrocytes were never observed.

In spite of their cytotoxicity, showed in KB cells, both EM and EA failed to induce micronuclei in the mice bone-marrow *in vivo*. The EA failure cannot be explained by impaired proerythroblast-polychromatic erythrocytes flow or PCE-NCE transition, followed by a decrease of PCE number in bone-marrow population (M. Hayashi et al. 1984; Hart and Hartley-Asp, 1983; Mirkova and Ashby, 1987). Neither were there evidences that bone-marrow became flooded with peripheral blood as a consequence of bone-marrow depression (Schmid, 1975). This rationale is supported by both the EA PCE/NCE normal rates and predominant micronuclei shape

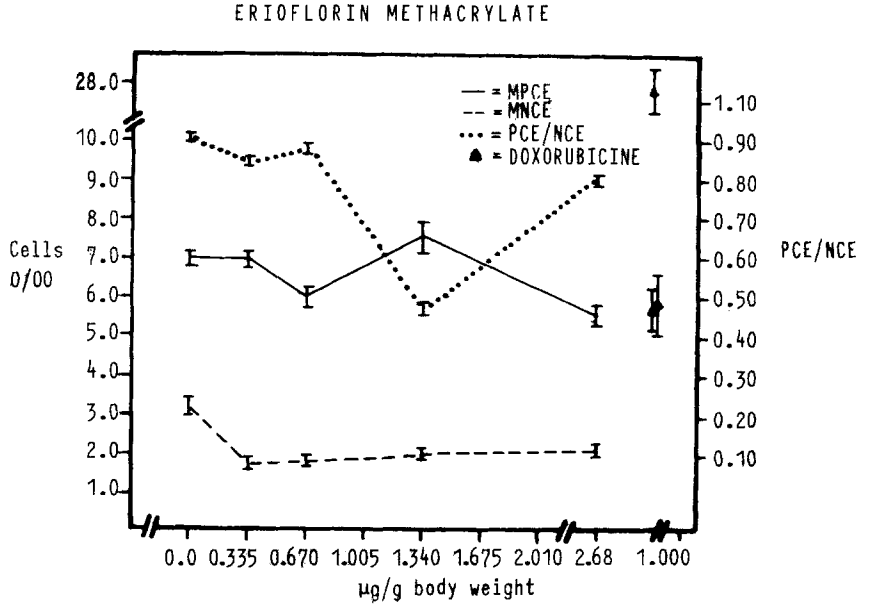

Figure 2. Dose-frequency relationship of micronuclei of mice erythrocytes treated with erioflorin methacrylate.

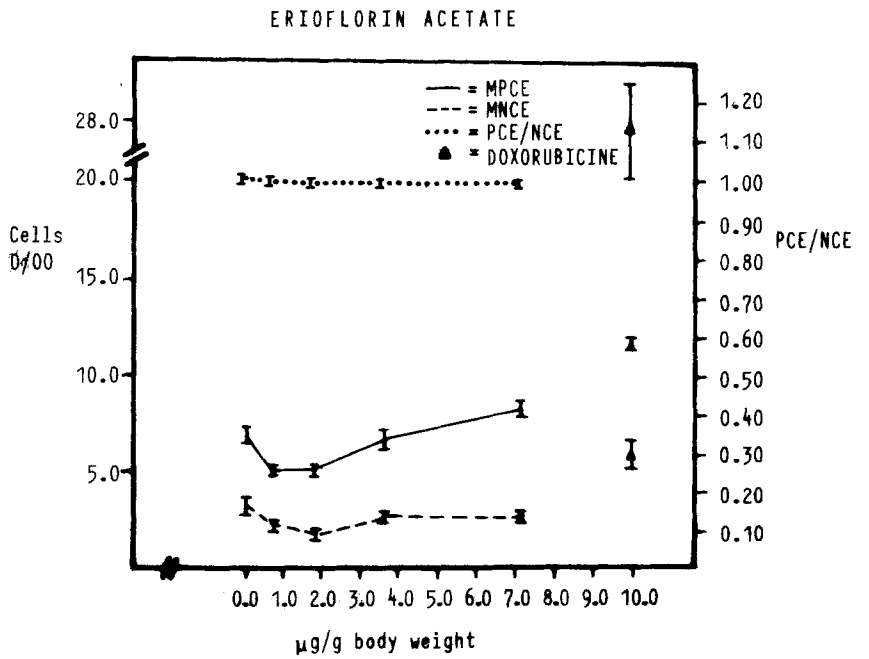

Figure 3. Dose-frequency relationship of micronuclei of mice erythrocytes treated with erioflorin acetate.

Table 1. Average values and standard deviations of quantitative analysis of bone-marrow erithrocytes from mice treated with erioflorin acetate and erioflorin methacrylate. (PCE=polychromatic erythrocytes; NCE=normochromatic erythrocytes; MPCE=micronucleated polychromatic erythrocytes; MNCE=micronucleated normochromatic erythrocytes; DOXO=doxorubicine;D-W=dioxan: water; EM=erioflorin methacrylate; EA=erioflorin acetate; b.wt.=body weight).

Doses µg/g b.wt.	Nº Cell Scored	PCE/animal	%PCE/animal	NCE/animal	%NCE/animal	MPCE/ animal	MPCE/ 1000PCE	MNCE/ animal	MNCE/1000NCE	PCE/NCE
DOXO 10.00	7060.00	2512.75	36.45±6.47	4547.45	63.54±6.47	69.25	27.57±4.33	25.25	5.78±1.45	0.58±0.16
D-W (1:10)	4988.25	2518.50	50.51±1.32	2469.75	49.49±1.32	17.50	6.94±0.82	8.00	3.25±0.92	1.02±0.05
EM 0.335	5154.25	2521.75	48.96±1.50	2632.50	51.04±1.50	17.50	6.93±0.80	4.50	1.73±0.59	0.95±0.05
EM 0.670	5139.00	2520.00	49.37±4.73	2619.00	50.63±4.73	15.00	5.94±1.03	4.75	1.80±0.39	0.98±0.18
EM 1.340	7046.00	2517.00	36.32±5.06	4529.00	63.77±5.06	19.00	7.54±1.65	9.00	2.00±0.59	0.57±0.12
EM 2.680	5316.75	2527.75	47.57±1.30	2789.00	52.43±1.30	14.00	5.53±1.12	6.25	2.23±0.25	0.90±0.04
EA 0.720	5062.75	2523.50	49.84±0.36	2539.25	50.15±0.36	12.75	5.04±0.66	5.50	2.15±0.48	0.98±0.01
EA 1.80	5079.25	2519.75	49.60±0.47	2559.50	50.39±0.47	12.75	5.05±0.87	4.75	1.85±0.35	0.98±0.01
EA 3.60	5091.75	2523.25	49.52±0.37	2568.50	50.48±37	14.50	6.73±1.24	6.50	2.53±0.52	0.98±0.01
EA 7.20	5091.00	2522.75	49.55±0.17	2568.25	50.44±0.17	18.25	7.23±1.02	6.50	2.53±0.50	0.98±0.008

Table 2. Mann-Whitney U test results of bone-marrow erythrocytes from mice treated with erioflorin methacrylate $\alpha = 0.05$. The > indicates that the effect of the left-column dose is greater than the correspondent effect of bottom-column dose. The Λ indicates that the effect of bottom-column dose is greater than the correspondent effect of left-colum dose.
(MPCE=micronuclei polychromatic erythrocytes; MNCE=micronuclei normochromatic erythrocytes; PCE=polychromatic erythrocytes; NCE=normochromatic erythrocytes; C-=negative control; C+= positive control).

	MNCE/1000 PCE					MNCE/1000 NCE					PCE/NCE				
C-	U=0 p=0.014 Λ					U=0 p=0.014 Λ					U=0 p=0.014 >				
0.335	U=0 p=0.014 Λ	U=7 p=0.443				U=0 p=0.014 Λ	U=1 p=0.029 Λ				U=0 p=0.014 >	U=5 p=0.243			
0.670	U=0 p=0.014 Λ	U=3 p=0.100	U=4 p=0.171			U=0 p=0.014 Λ	U=0 p=0.014 Λ	U=8 p=0.557			U=0 p=0.014 >	U=4 p=0.171	U=8 p=0.557		
1.340	U=0 p=0.014 Λ	U=6 p=0.343	U=5 p=0.243	U=1 p=0.029 >		U=0 p=0.014 Λ	U=0 p=0.057	U=6 p=0.343	U=7 p=0.443		U=7 p=0.443	U=0 p=0.014 Λ	U=0 p=0.014 Λ	U=0 p=0.014 Λ	
2.680	U=0 p=0.014 Λ	U=2 p=0.057	U=2 p=0.057	U=5 p=0.243	U=4 p=0.171	U=0 p=0.014 Λ	U=2 p=0.057	U=7 p=0.443	U=5 p=0.243	U=5 p=0.243	U=0 p=0.014 >	U=0 p=0.014 Λ	U=2 p=0.057	U=4 p=0.171	U=0 p=0.014
DOSES	C+	C-	0.335	0.670	1.340	C+	C-	0.335	0.670	1.340	C+	C-	0.335	0.670	1.340

Table 3. Mann-Whitney U test results of bone-marrow erythrocytes from mice treated with erioflorine acetate $\alpha = 0.05$. The > indicates that the effect of the left-column dose is greater than the correspondent effect of bottom-column dose. The Λ indicates that the effect of the bottom-column dose is greater than the correspondent effect of left-column dose. (MPCE=micronuclei polychromatic erythrocytes; MNCE=micronuclei normochromatic erythrocytes; PCE=polychromatic erythrocytes; NCE=normochromatic erythrocytes; C-=negative control; C+=positive control).

	MNCE/1000 PCE					MNCE/1000 NCE					PCE/NCE				
C-	U=0 p=0.014 Λ					U=0 p=0.014 Λ					U=0 p=0.014 >				
0.72	U=0 p=0.014 Λ	U=0 p=0.014 Λ				U=0 p=0.014 Λ	U=1 p=0.029 Λ				U=0 p=0.014 >	U=2 p=0.057			
1.80	U=0 p=0.014 Λ	U=0 p=0.014 Λ	U=7 p=0.443			U=0 P=0.014 Λ	U=0 p=0.014 Λ	U=3 p=0.100			U=0 p=0.014 >	U=2 p=0.057	U=4 p=0.171		
3.60	U=0 p=0.014 Λ	U=6 p=0.343	U=1 p=0.029 >	U=1 p=0.029 >		U=0 p=0.014 Λ	U=4 p=0.171	U=5 p=0.243	U=2 p=0.057		U=0 p=0.014 >	U=2 p=0.057	U=6 p=0.343	U=7 p=0.443	
7.20	U=0 p=0.014 Λ	U=8 p=0.557	U=0 p=0.014 >	U=0 p=0.014 >	U=5 p=0.243	U=0 p=0.014 Λ	U=3 p=0.100	U=6 p=0.343	U=2 p=0.057	U=8 p=0.557	U=0 p=0.014 >	U=2 p=0.057	U=3 p=0.100	U=8 p=0.557	U=7 p=0.443
DOSES	C+	C-	0.72	1.80	3.60	C+	C-	0.72	1.80	3.60	C+	C-	0.72	1.80	3.60

and size, which are not in correspondence with micronuclei characteristics induced by antimitotic drugs (Yamamoto and Kikushi, 1980). Similar conclusions are applicable to the EM results obtained in animals treated with EM 1/2LD50 and LD50 doses for KB cells. Nevertheless, the results obtained with the two higher doses of EM show that the EM behaviour at both 2LD50 and 4LD50 doses for KB cells, is clearly disturbing to bone-marrow cell population.

We do not know which pharmacokinetic, molecular, tissue-specific metabolic activation, and cell proliferation factors or time-response patterns could be operating on the EM and EA bone-marrow action. These questions remain unanswered, but work directed toward answering these questions is currently in progress in our laboratory. The fact that both EM and EA treatments exhibit reduced incidences of MNCE lead us to think that preexisting micronucleated cells are more sensitive to these chemicals than normal cells. This fact could entail important therapeutical implications.

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